Interrogating centrosome protein dynamics, centriolar satellite regulation mechanisms, and autofluorescence characterization of Caenorhabditis elegans using Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) and fluorescence microscopy

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Interrogating centrosome protein dynamics, centriolar satellite regulation mechanisms, and autofluorescence characterization of *Caenorhabditis elegans* using Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) and fluorescence microscopy

By

Elizabeth Cameron

Accepted in Partial Completion of the Requirements for the Degree Master of Science

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Master’s Thesis

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Elizabeth Cameron

March 1, 2023
Interrogating centrosome protein dynamics, centriolar satellite regulation mechanisms, and autofluorescence characterization of *Caenorhabditis elegans* using Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) and fluorescence microscopy

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Elizabeth Cameron
March 1, 2023
Abstract

Centrosomes are required for human cells to divide and differentiate into the many different embryonic tissues that form throughout development. Mutations that drastically disrupt centrosomes cause embryonic lethality and cancer (Breslow and Holland, 2019). More subtle mutations cause congenital birth defects including blindness, olfactory deficits, and limb, heart, and brain malformations (Breslow and Holland, 2019). PCNT is an essential human gene that encodes for the centrosome protein Pericentrin (Breslow and Holland, 2019). Pericentrin organizes the structure of the centrosome by serving as a scaffold protein (Breslow and Holland, 2019; Delaval and Doxsey, 2010). Pericentrin also interacts with other centrosome proteins, which play a role in centrosome-mediated microtubule formation. Additionally, Pericentrin recruits enzymes that are involved in centrosome duplication and maturation, including the critical centrosome regulating enzyme, Aurora-A kinase. Although Pericentrin recruits Aurora-A, it is not clear whether Pericentrin regulates Aurora-A kinase activity. To test this possibility, single molecule imaging of Aurora-A activity in live cells was accomplished using Förster Resonance Energy Transfer (FRET) quantified with Fluorescence Lifetime Imaging Microscopy (FLIM; FRET-FLIM).

Pericentrin exists in two separate populations within cells. One population resides at the centrosome, and a second population, known as satellites, exist as dynamic granules that traffic to and from the centrosome (Liu et al., 2010). Populations of these satellites are found at or near both the centrosome and the Golgi apparatus. Additionally, the satellites mimic the distribution pattern of the Golgi throughout the cell cycle. During interphase, the satellites cluster at both the centrosome and Golgi. However, by mitosis the satellites and the Golgi are both nearly fully dispersed and are spread evenly throughout the entirety of the cell. To observe potential effects of changes in Golgi morphology on satellites, the Golgi was broken apart into small vesicles using the Golgi disruptor drug Brefeldin A. Using this methodology, we determined that Golgi morphology plays a role in the distribution of centriolar satellites by causing Golgi-associated satellites to disperse alongside the Golgi.

Capitalizing on the FLIM capabilities of the Stellaris, we set out to characterize autofluorescence of C. elegans using FLIM microscopy. The model organism C. elegans has autofluorescence that is problematic for biological imaging assays. The autofluorescence spectrum and fluorescence lifetime characterization of C. elegans autofluorescence is currently unknown. We characterized the autofluorescence emission spectra at four excitation wavelengths, 405, 473, 561 and 647 nm. Of these spectral scans the green species (473 nm excitation) of autofluorescence overlaps with the commonly used fluorescent protein GFP’s spectra. However, by utilizing FLIM, we determined that the green autofluorescence can be easily separated from GFP fluorescence due to their different fluorescence lifetime properties. The separation of lifetime between the autofluorescence and GFP fluorescence improves the ability to detect and quantify GFP fluorescence in C. elegans cilia.

Overall, this work provides a foundation for future experiments to determine effects of Pericentrin overexpression on Aurora-A kinase, a model of regulation of centriolar satellites via the Golgi, as well as a novel method for characterizing autofluorescence using FLIM.
Acknowledgements

I am grateful for the immense amount of support I have received for this project. I am especially grateful for the mentorship I have received from my advisor, Dr. Nick Galati. I appreciate the time and energy you have dedicated to not only my project, but to helping me become the scientist I am today. I’m grateful you took the risk of having me be one of your first graduate students!

I would also like to thank my committee members Dr. Jeanine Amacher, Dr. Steven R. Emory, and Dr. Dan Pollard for their guidance, suggestions, and support throughout this project. I want to give a special shout out to Dr. Emory for allowing me to also TA for the Leica through STS.

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Lastly, thank you to all of my family and friends for encouraging me to pursue my passions and supporting me along the way. I would not have made it through this without you and am lucky to have each and every one of you be a part of my life.

I couldn’t have done this without all of you!
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Chapter 1: Interrogating dynamic changes in centrosome protein activity using FRET-FLIM and fluorescence microscopy

Introduction

A.1. Centrosomes are important because they organize microtubules for essential cellular processes. Centrosomes are evolutionarily ancient structures that play diverse roles in the cell (Bornens, 2012). Centrosomes are essential for human development due to their roles in cell division, cilia assembly, orienting microtubule arrays, organizing the mitotic spindle, and influencing cell shape, polarity, and motility (Darling et al., 2017). Mutations in genes that code for centrosome proteins cause congenital birth defects, degenerative tissue disorders (Joukov and De Nicolo, 2019), and tumorigenesis (Bettencourt-Dias et al., 2011). Many of these mutations disrupt the 3-dimensional conformation (i.e., shape) and/or the localization of proteins at the centrosome (Gupta et al., 2015). However, due to the limited resolution of the light microscope, it has been difficult to investigate the relationship between centrosome protein shape and localization.

Cilia are structures that extend outward from centrioles located in the center of the human centrosome (Figure 1). Cilia play a key role in information signaling (Nigg and Raff, 2009) and are often assembled from centrioles that migrate to the cell surface in non-proliferating cells (Nigg and Raff, 2009). For example, olfactory neurons contain 15-30 sensory cilia (McEwen et al., 2008) that are responsible for our sense of smell (Falk et al., 2015). Our sense of sight depends on cilia that act as a transport system to connect the light sensitive outer segment of the photoreceptor cell to the inner metabolizing segment of the photoreceptor (Falk et al., 2015). During development, cilia are essential for vertebrate cell differentiation via
Figure 1. Structure of centrosome composed of centrioles and PCM. Adapted from Lawo et al., 2012 Subdiffraction imaging of centrosomes reveals higher-order organizational features of pericentriolar material. Nat Cell Biol 14, 1148–1158. Centrosome structure with components, including PCNT (Pericentrin) in a toroid shape. Elongated proteins making up the PCM surround the central parent centriole.
Hedgehog (Hh) signaling (Breslow and Holland, 2019). The primary cilium regulates Hh signaling by both activating and suppressing the pathway (Wong et al., 2009). The activation relies on the proto-oncogene Smoothened (Smo) trafficking to the primary cilium, while negative regulation relies on cilium-dependent proteolytic processing of Gli3 into a form which can repress the Hh pathway (Gli3-R). The regulation of the Hh pathway is important for mammalian organogenesis, regeneration, and homeostasis. Additionally, elevated activity of the Hh pathway is associated with tumor formation and diverse types of cancers (Wong et al., 2009). Collectively, these examples provide strong evidence that centrosome-mediated cilia formation is critical for human development and sensation.

A.2. **Centrosome duplication is coordinated through the cell cycle.** At the start of the cell cycle, each human cell contains one centrosome inherited from the prior dividing cell (Figure 2) (Breslow and Holland, 2019). Centrosomes are made up of two centrioles (Breslow and Holland, 2019) (Figure 2) and each centriole is surrounded by a toroid (donut) of pericentriolar material (PCM) (Figure 1). The two centrioles are connected by a flexible linker, forming a single centrosome (Lüders, 2012) (Figure 2).

During G1 the inherited centrosome undergoes centriole disengagement, where the centrioles separate from one another. Additionally, the primary cilium is formed from the centriole during this phase so that it can sense and relay extracellular cues to the cell (Joukov and De Nicolo, 2019). By S phase the two centrioles begin duplicating, becoming “parents” to newly forming pro-centrioles and the primary cilium is disassembled (Breslow and Holland, 2019). By G2, the pro-centrioles begin elongation and maturation until they are fully formed, now referred to as “child” centrioles. Once the centrosomes have matured, they begin to separate to opposite sides of the cell to orient the mitotic spindle during cell division (Holland et al., 2010). After
Figure 2. Centrosome Duplication Cycle. Schematic representation of centrosome duplication. G1: Single centrosome present inherited from previous cell during division. S: Parent centrosome begins duplicating resulting in one parent and one child centriole. G2: Centrosome maturation occurs by recruiting other proteins and factors to the centrosome. Centrioles elongate by lengthening their microtubules. M: Centrosome disengagement. Created with BioRender.com.
mitosis and before the start of G1, the centrioles disengage. The disengagement prepares the centrioles for the next round of primary cilium formation and centrosome duplication (Karki et al., 2017). Without centrosomes, cells activate cell cycle checkpoints, which result in cell cycle arrest or cell death (Holland et al., 2012) and an inability to produce cilia. Conversely, cells that have too many centrosomes produce too many cilia (Mahjoub and Stearns, 2012) and have altered polarity/symmetry. The presence of additional cilia causes altered Shh signaling and additional centrosomes cause aneuploidy due to improper segregation of chromosomes and other cellular components (Holland et al., 2012). Therefore, understanding the molecular processes that underlie centrosome-mediated cilia formation and centrosome duplication is essential for understanding cell cycle progression and homeostasis.

**A.3. Pericentrin regulates centrosome formation and function.** Pericentrin is a large coiled-coil protein that is a multifunctional scaffold for anchoring proteins and protein complexes to the PCM at the base of each centriole (Breslow and Holland, 2019; Delaval and Doxsey, 2010). Pericentrin’s C-terminal PACT domain localizes Pericentrin to the centrosome (Gillingham and Munro, 2000), where Pericentrin forms an elongated linear filament with the C-terminus bound to the wall of the centriole and the N-terminus extending outward away from the centriole (Breslow and Holland, 2019). This elongated structure allows Pericentrin to interact with other centrosome proteins, such as γ-tubulin and CDK5RAP2, which play critical roles in centrosome-mediated microtubule formation. Mutations in the gene that encodes for Pericentrin are associated with Seckel syndrome (Griffith et al., 2008) and Majewski/microcephalic osteodysplastic primordial dwarfism type II (MOPDII) (Rauch et al., 2008) and can disrupt PCNT association with other centrosome components such as Cep57 (Watanabe et al., 2019). Mice with disruptions in the PCNT gene (Miyoshi et al., 2009), causing reduced levels of
Pericentrin, result in embryos with phenotypic similarities to humans with primordial dwarfism including short stature and microcephaly (unpublished data; Akbarian, S., C. Lo, and G. Zheng, personal communication). Conversely, individuals with Trisomy 21 have three copies of chromosome 21, which contains the *PCNT* gene (Galati et al., 2018). This causes elevated Pericentrin levels and disrupted cilia assembly and Shh signaling. Collectively, these data suggest that the precise regulation of Pericentrin expression plays a role in the proper development of cells.

**A.4. Pericentrin interacts with and localizes Aurora-A kinase.** Beyond recruiting structural proteins and microtubule-organizing proteins, another major function of Pericentrin is to recruit enzymes that control centrosome duplication and maturation (Gillingham and Munro, 2000). One such enzyme is Aurora-A kinase, which is a serine/threonine kinase that localizes to the centrosome (Joukov and Nicolo, 2018). Aurora-A kinase activity fluctuates during the cell cycle, with high activity during G1 and mitosis, and little to no activity during S phase (Bertolin et al., 2016). Aurora-A kinase regulates centrosome function, mitotic spindle formation, and Shh signaling. Malfunctions in the enzyme can lead to checkpoint defects, aneuploidy, genetic instability, and transformation (Karthigeyan et al., 2011). Additionally, increased levels of Aurora-A kinase and increased Aurora-A kinase activity are seen in many cancers associated with solid tumor formation (Landen et al., 2007). Interestingly, those with Trisomy 21 have remarkably low rates of solid tumor formation (Hasle et al., 2016). Since Pericentrin interacts with Aurora-A kinase (Joukov et al., 2014), it is possible that Pericentrin overexpression decreases solid tumor formation by modulating Aurora-A kinase activity in and around the centrosome. Single molecule imaging of Aurora-A in live cells is necessary to test the possibility that expression of Pericentrin alters Aurora-A activity. Testing this possibility can be
accomplished using Förster resonance energy transfer, or FRET, and quantified with fluorescence lifetime imaging microscopy, or FLIM (Becker, 2012).

A.5. FRET-FLIM can be used to investigate protein interaction, activity, and conformation.

The FRET-FLIM technique allows for the visualization and quantification of dynamic intramolecular and intermolecular interactions using donor/acceptor pairs of fluorescent molecules (Becker, 2012). When a donor is within 10 nm of its paired acceptor molecule, the donor will excite the acceptor through energy transfer (Figure 3) (Ghisaidoobe and Chung, 2014). Typically, FRET is measured as an increase in the fluorescence intensity of the acceptor. However, this approach is difficult due to the need to correct for the bleed through of donor emission photons into the acceptor channel. In contrast, FLIM is a quantitative approach to FRET that measures the temporal decay of single photons emitted by the donor. Due to photophysical effects that occur during FRET, the temporal decay of donor photons emitted during FRET is faster than the temporal decay of donor photons emitted in the non-FRET state. Therefore, FLIM allows FRET to be quantified as a single quantitative lifetime measurement of the donor without the need to unmix donor emission photons and acceptor emission photons.

FRET biosensors, or molecules tagged with paired fluorescent molecules, have previously been used to study conformational changes of proteins within live cells. This has allowed for visualization and quantification of conformational changes and stressors affecting proteins at any point during the cell’s lifecycle (Ng et al., 1999). An Aurora-A Kinase FRET-FLIM intramolecular biosensor that contains GFP on the N terminus and mCherry on the C terminus has been previously utilized to investigate Aurora-A kinase enzyme activity (Figure 4) (Bertolin et al., 2016). Similarly, FRET has also been used to analyze intermolecular protein interactions by tagging separate proteins with paired fluorescent molecules to determine
Figure 3. Occurrences of FRET dependent on fluorescent molecule distance. Schematic of distance of fluorescent molecule pairs effect on FRET occurrence. Panel A shows a molecule in a linear conformation with a distance between the fluorescent tagged termini of greater than 10 nm. In this case, the fluorescent molecules are too far apart to allow for energy transfer, therefore no FRET will occur. However, panel B with a molecule in a closed conformation with fluorescent tagged termini at or within 10 nm of one another is at a distance, which will enable the occurrence of energy transfer between the green donor molecule and red acceptor. Therefore, the molecule will FRET. In a FRET state lifetime is shortened due to energy transfer, while in a non-FRET state lifetime is longer. Created with BioRender.com
Figure 4. Representative fluorescence and lifetime of GFP-AURKA-mCherry cells. Figure from Bertolin et al., 2016 *A FRET biosensor reveals spatiotemporal activation and functions of aurora kinase A in living cells*. Nature Communications 7, 12674. Intensity images (top panels) with their compared lifetime images (bottom panels) for AuroraA kinase biosensor cells. Lifetime does not correspond to intensity of the fluorescent protein and rather measures a fluorophore's time spent in an excited state. During G1 and mitosis the AuroraA kinase biosensor has a short lifetime indicating it is in an active state as seen by the small intensely yellow dots in squares D and F. During S phase longer lifetime is observed in panel E with the longest observed lifetime at the centrosomes.
potential interactions between the two proteins. For example, the intermolecular FRET technique has previously been used to study the interaction between G proteins and their regulators (G\textsubscript{ia1} and RGS14) at centrosomes in \textit{Drosophila melanogaster} (Cho and Kehrl, 2007). Collectively these examples show that both \textit{intramolecular} and \textit{intermolecular} FRET can be used to investigate enzymatic activity and protein-protein interactions.

\textbf{A.6. Does Pericentrin expression effect Aurora-A activity?} An Aurora-A Kinase FRET Biosensor (Bertolin et al., 2016) was used to determine if changes in Pericentrin expression alter Aurora-A activity, localization, or function within live mammalian cells. By understanding how changes in expression levels of proteins associated with the Aurora-A Kinase pathway may affect Aurora-A activity or localization, we may better understand the regulatory mechanisms for the pathway. As Aurora-A is a cancer therapeutic target, discovering potential regulators of the Aurora-A pathway has great implications for human health developments and future cancer therapies.
Materials and Methods

B.1. Cell culture methods. NIH 3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum (CS) and penicillin, streptomycin and fungizone to prevent contamination. Hek293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin, streptomycin and fungizone. U2OS cells were cultured in McCoys 5A Medium supplemented with 10% fetal bovine serum (FBS) and penicillin, streptomycin and fungizone. All cells were kept at 37°C in a 5% CO$_2$ environment.

B.2. Plasmid Preparation and Transient Transfection Protocol. All plasmids were streaked on appropriate antibiotic plate and incubated for 16 hours. Colonies were isolated and restriction enzyme digests were run for each plasmid to confirm plasmid identity. Plasmid DNA was prepped according to the Zymo Zippy miniprep kit protocol.

Coverslip Preparation- Cells were passaged onto 50 mm glass bottom coverslip dishes (MatTek P50G-1.5-14F 50 mm Uncoated γ-irradiated) between 20-30% confluency into 250 uL of appropriate cell culture media. Cells were allowed to grow on the coverslips for 24-48 hours before transfection to ensure adherence of cells to the coverslips. Coverslips were kept at 37°C in a 5% CO$_2$ environment after plating.

JetPRIME® Transfection Protocol- Coverslips were transfected when cells reached between 40-60% confluency using the JetPRIME® transfection kit (Polyplus) and appropriate plasmid DNA. Cells were incubated in media containing transfection agent and DNA for 6 hours. Media containing transfection reagent and plasmid DNA was then removed and replaced with appropriate cell culture media dependent upon cell type. After the washout cells were left to
incubate overnight before imaging. JetPRIME® reagent was used for transfection in NIH 3T3 cells and Hek293T cells.

Viafect™ Transfection Protocol- Coverslips were transfected when cells reached approximately 40-60% confluency. Appropriate plasmid DNA was added to serum free media with transfection reagent and cells were incubated in the mixture for 24 hours before imaging. Viafect™ transfection protocol was used for transfection in U2OS cells.

B.3. Pharmacology. Cell cycle synchronization- Cells were synchronized in G1 by a 1% serum starvation for 24-48 hours. Cells were synchronized at the G1/S boundary by incubating the coverslips in 5 ng/μL of +/- Aphidicolin (Adipogen) for 18-20 hours before imaging. Cells were synchronized in G2/M by incubating the cells in 100 ng/mL nocodazole (Cayman Chemicals) for 18 hours. Before the cells were imaged, the drug was washed out of the cells and cells were left to incubate for two hours to allow the cells to reach S phase (Bertolin et al., 2016).

Aurora-A Inhibition- MLN8237 (Cayman Chemicals) inhibits autophosphorylation of Thr288, which prevents the kinase activity of Aurora-A. This kinase activity is what contributes to the Aurora-A Biosensor being in a FRET/Non-FRET state. MLN8237 was reconstituted in DMSO and used at a final concentration of 50 nM in the live cell imaging chamber to inhibit Aurora-A kinase activity.

B.4. FRET Controls. To establish a baseline for FRET efficiency and lifetime within live cells three major controls were utilized (Figure 5). The first control, pcDNA3.1 CMV mCherry-eGFP BGH, expresses eGFP and mCherry separated by a linker that is designed to be exactly 10 nm in length (Figure 6). This plasmid expresses a protein that serves as a positive control with consistent high FRET activity (Table 1 & Figure 6) (Octeau et al., 2018).
Table 1. Control and experimental plasmids with respective fluorescent tag and function

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Parent Vector</th>
<th>Promoter</th>
<th>Residues</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEGFP-N1</td>
<td>mEGFP-N1</td>
<td>CMV</td>
<td>mEGFP</td>
<td>FRET baseline control for establishing donor lifetime baseline.</td>
</tr>
<tr>
<td>mCherry2-N1</td>
<td>mCherry2-N1</td>
<td>CMV</td>
<td>mCherry2</td>
<td>FRET baseline control for establishing acceptor lifetime baseline and interaction with donor.</td>
</tr>
<tr>
<td>pcDNA3.1 CMV mCherry-eGFP BGH</td>
<td>pcDNA3.1</td>
<td>CMV</td>
<td>Tandem mCherry-eGFP fusion</td>
<td>FRET 10 nm linker control construct as baseline continuous FRET control.</td>
</tr>
<tr>
<td>pAuroraA-GFP-AURKA</td>
<td>pEGFP-C1</td>
<td>AURKA</td>
<td>GFP-AURKA (H. sapiens)</td>
<td>Aurora-A kinase with GFP tag for establishing baseline donor lifetime for Aurora-A biosensor.</td>
</tr>
<tr>
<td>pAuroraA-GFP-AURKA-mCherry</td>
<td>pEGFP-C1</td>
<td>AURKA</td>
<td>GFP-AURKA-mCherry (H. sapiens)</td>
<td>Aurora-A kinase biosensor tagged with GFP and mCherry to determine Aurora-A baseline activity through cell cycle.</td>
</tr>
<tr>
<td>pAuroraA-GFP-AURKA_K162M-mCherry</td>
<td>pEGFP-C1</td>
<td>AURKA</td>
<td>GFP-AURKA K162M-mCherry (H. sapiens)</td>
<td>Kinase dead Aurora-A biosensor to be used as an inactivated control for baseline activity and FRET efficiency.</td>
</tr>
</tbody>
</table>
Figure 5. Model of cytoplasmic FRET controls. The maximum FRET control (A) will continuously FRET throughout the cell due to the non-specificity of the molecule and close proximity of the fluorescent molecules. The donor only control (B) will have a longer lifetime compared to the FRET linker. The donor and acceptor separated control (C) should have the same lifetime as the donor only control to show that mEGFP and mCherry as separate molecules do not FRET.
Figure 6. Plasmid map of pcDNA3.1 CMV mCherry-eGFP BGH. Plasmid for mCherry-eGFP connected by 10 nm flexible linker protein expression within cells. Used as positive FRET control due to the molecule being in a constant FRET state. Sequence from Addgene.
The second control, mEGFP-N1, expresses mEGFP that acts as the donor only FRET negative control that is in a constant non-FRET state (Figure 5 & Figure 7). The third control mCherry2-N1, which expresses the FRET acceptor mCherry, will serve as an additional negative control to ensure the donor and acceptor molecules do not FRET when simultaneously expressed within cells (Figure 5 & Figure 8). Expression of these proteins in live cells ensures differences in FRET efficiency can be measured between the donor only and donor-acceptor pair within live cells, which has not been previously established on the Leica Stellaris 8 Falcon at WWU. All control plasmids were transfected into mammalian cell lines (NIH 3T3/Hek293T) using either JetPRIME® transfection reagent or Viafect™ transfection reagent. JetPRIME® buffer and transfection reagent were used for transfection of all FRET control plasmids to establish FRET-FLIM capabilities on the Stellaris 8 Falcon in NIH 3T3 cells. As cell lines changed to adapt to circumstances with the Aurora-A biosensor, control plasmids were transfected into each cell type using an appropriate transfection reagent and baselines were re-established for the controls.

**B.5. Aurora-A Kinase Biosensor Baseline FRET Establishment.** Baseline FRET efficiency for the Aurora-A kinase biosensor, pAurora-A-GFP-AURKA-mCherry (Table 1 & Figure 9) (Bertolin et al., 2016), was established on the Leica Stellaris 8 Falcon. The Aurora-A kinase biosensor was transfected into mammalian cells (NIH 3T3, Hek293T, and U2OS) using either jetPRIME® buffer and transfection reagent (3T3, Hek293T, U2OS), or ViaFect™ transfection reagent (U2OS) and imaged at multiple points during the cell cycle (G1, S, mitosis), which corresponded to prior points of published activity/non-activity of the enzyme (Bertolin et al., 2016). A donor only version of the biosensor, pAuroraA-GFP-AURKA
Figure 7. Plasmid map of mEGFP-N1. Plasmid for mEGFP protein expression within cells. Used as donor only negative FRET control. Sequence from Addgene.
**Figure 8. Plasmid map of mCherry2-N1.** Plasmid for mCherry protein expression within cells. Used alongside mEGFP plasmid as a negative FRET control. Sequence from Addgene.
**Figure 9. Plasmid map of pAuroraA-GFP-AURKA-mCherry.** Plasmid for Aurora-A biosensor. Includes both FRET donor and acceptor proteins with ability to change conformation to bring proteins close enough together to FRET dependent upon cell cycle stage. Sequence from Addgene.
Figure 10. Plasmid map of pAuroraA-GFP-AURKA. Plasmid for Aurora-A-GFP protein expression. Used as donor only negative FRET control for the Aurora-A biosensor. Sequence from Addgene.
Figure 11. Plasmid map of pAuroraA-GFP-AURKA_K162M-mCherry. Plasmid for kinase dead version of the Aurora-A biosensor which doesn’t allow for phosphorylation of the Aurora-A biosensor. Includes both FRET donor and acceptor proteins but lacks ability to change conformation to bring proteins close enough together to FRET. Sequence from Addgene.
(Table 1 & Figure 10) and a kinase dead version of the Aurora-A kinase biosensor, pAurora_K162M biosensor, were also transfected into mammalian cells (NIH 3T3 or Hek293T) and used as negative controls for the active Aurora-A kinase biosensor (Table 1 & Figure 11). The selective Aurora-A inhibitor, MLN8237 was also used as a secondary negative control to inhibit Aurora-A biosensor function at a final concentration of 50 nM.

Due to observing lifetimes that did not match the published expected value ranges in Aurora-A kinase biosensor expressing NIH 3T3 cells, the biosensor was then transfected into HEK293T cells using the jetPRIME® buffer and transfection reagent. However, the cells did not properly express the Aurora-A Biosensor with lack of localization at centrosomes. To replicate Bertolin et al., 2016 methodology more closely after attempts at other methodology, the biosensor was transfected into U2OS cells using ViaFect™ transfection reagent.

**B.6. FRET-FLIM Microscopy.** Transfected cells were imaged with a 63X 1.2 numerical aperture water immersion objective attached to an automated Leica Stellaris 8 laser scanning confocal microscope. Samples were placed into an environmental control chamber mounted on to the stage of the fully automated microscope at 37°C in a 5% CO₂ environment. eGFP was excited with the 488 nm laser line of an 80 MHz pulsed white light laser with varying laser intensity to keep the photon/pulse ratio at or near 0.8 and maximum pixel intensity near 200 photons/pixel. Emission photons were collected using a time-correlated single photon counting device between 494–581 nm. Photon arrival times were analyzed using the Phasor approach through the Falcon - FLIM module of the Leica LasX software package, which provides a simple 2D graphical view of the distribution of observed lifetimes, separating different lifetime populations without curve fitting by plotting each individual lifetime per pixel from the observed image onto the phasor plot (Ossato, 2019). Secondary analyses of lifetime for the Aurora-A
Kinase Biosensor were performed using both the curve fitting approach through the Falcon-FLIM module of the Leica LasX software and Fast-FLIM average photon arrival through ImageJ. The Fast-FLIM approach utilized Fast-FLIM images produced by the LasX FLIM module. Fast-FLIM images take into account the average lifetime of all photons per pixel and create a color-coded image based upon the average pixel lifetime. The images were exported into ImageJ software where regions of interest were created by thresholding out low intensity pixels within the intensity image and then creating an overlay onto the Fast-FLIM image. Thresholding left behind pixels at or near the centrosomes and mitotic spindle where photon count per pixel was highest within the image. Within the regions of interest, pixel lifetimes were averaged resulting in a value for the average lifetime of the region of interest.
Results and Discussion

C.1. A FRET-FLIM assay was established to investigate intramolecular protein interactions.

To determine a FRET baseline, a series of cytoplasmic controls were established in NIH 3T3 cells (Figure 5). The first control established the maximum detectable FRET efficiency in the cytosol with a 10 nm long linker connecting a C-terminal mCherry tag and an N-terminal GFP tag (Figure 12). A plasmid encoding this construct was transfected into mammalian NIH/3T3 cells and FRET efficiency of the expressed protein was analyzed using Phasor FLIM with an average lifetime of 1.880 ns (Figure 12). The two additional cytoplasmic control conditions established the baseline lifetime for GFP (donor) (Figure 13) and the possibility of any intermolecular interactions between GFP (donor) and mCherry (acceptor) molecules if present within the same cell (Figure 14).

A difference in lifetime was observed between cells expressing GFP (Figure 13) or cells expressing both GFP and mCherry as separate proteins (negative FRET controls) (Figure 14) when compared to the 10 nm mCherry/eGFP linker (positive FRET control) (Figure 12). The negative control conditions have an average lifetime of approximately 2.3 ns (Figure 15) and the positive control has an average lifetime of approximately 1.9 ns (Figure 15). The difference in lifetime between the negative control and positive control conditions is approximately 445 picoseconds, indicating occurrence of FRET for the positive control and lack of FRET for both negative controls (Figure 15). (Llères et al., 2007). Collectively, this data demonstrates that intramolecular FRET can be detected, and FRET efficiency can be quantified using phasor FLIM.
Figure 12. Cytoplasmic FRET Linker Positive Control Establishment. Lifetime images of cytoplasmic FRET positive control in live NIH 3T3 cells. Each pixel on the image (512x512) represents single lifetime measurement within the image with lifetime range denoted (left). Phasor plot for individual cell. Phasor plot contains all pixels from lifetime image plotted on unit semicircle. Lifetime value of cell denoted on image where longer lifetimes are associated with the left range of the unit semicircle and shorter lifetimes with the right range. Lifetime value for cell contained within red circle on plot.
**Donor Only Negative FRET Control**

*Figure 13. Cytoplasmic FRET Donor Only Negative Control Establishment.* Lifetime images of cytoplasmic mEGFP donor only control in live NIH 3T3 cells. Each pixel on the image (512x512) represents single lifetime measurement within the image with lifetime range denoted (left). Phasor plots for individual cell. Phasor plot contains all pixels from lifetime image plotted on unit semicircle. Lifetime value of cell denoted on image where longer lifetimes are associated with the left range of the unit semicircle and shorter lifetimes with the right range. Lifetime value for cell contained within red circle on plot.
Figure 14. Cytoplasmic FRET Donor and Acceptor Separated Negative Control

Establishment. Lifetime images of cytoplasmic mEGFP donor and mCherry acceptor both expressed within live NIH 3T3 cells, but not linked to one another as with the FRET linker positive control. Each pixel on the image (512x512) represents single lifetime measurement within the image with lifetime range denoted (left). Phasor plots for individual cell. Phasor plot contains all pixels from lifetime image plotted on unit semicircle. Lifetime value of cell denoted on image where longer lifetimes are associated with the left range of the unit semicircle and shorter lifetimes with the right range. Lifetime value for cell contained within red circle on plot.
Figure 15. Confirmation of cytoplasmic FRET control establishment. (A). Phasor plots of average lifetime compared between mEGFP-N1 and pcDNA3.1 CMV mCherry-eGFP expressing cells. N=20 cells per condition (mEGFP-N1 and pcDNA3.1 CMV mCherry-eGFP). Average lifetime per condition denoted on phasor plots. Average lifetime comparison is an overlay of the mEGFP-N1 and pcDNA3.1 CMV mCherry-eGFP phasors from (A) where two distinct lifetime populations are observed. pcDNA3.1 CMV mCherry-eGFP has a shorter lifetime compared to mEGFP-N1 and the position of two distinct populations indicates the occurrence of FRET. (B). Box plot of observed lifetimes per condition with average lifetime table. N=20 cells per condition. (C) Average lifetime of pcDNA3.1 CMV mCherry-eGFP BGH transfected cells (1.904 ±0.1234 ns) were observed to be lower than mEGFP-N1 (2.322 ±0.0342 ns) and mEGFP-N1 + mCherry2-N1 (2.349 ±0.0308 ns).
C.2. Detecting Aurora-A kinase activity using a FRET biosensor. To determine the baseline expression and lifetime of the Aurora-A FRET biosensor, the biosensor was transfected into NIH 3T3 cells. The Aurora-A FRET biosensor was found to locally express at centrosomes in NIH 3T3 cells after transient transfection (Figure 16A). However, the lifetime values obtained via phasor FLIM for cell cycle stages G1, S, and mitosis did not match the previously reported expected lifetime ranges, with no observable pattern of lifetime per cell cycle stage (Figure 16B & 16C) (Bertolin et al., 2016). Additionally, a kinase dead version of the Aurora-A biosensor, which should be in a constitutive non-FRET state, had variable lifetime values with no observable pattern (Figure 16B & 16D).

As the biosensor was created and utilized within human U2OS cells by the original authors, the Aurora-A biosensor was transfected into human HEK293T cells and subsequently in U2OS cells. While proper expression of the cytoplasmic FRET controls was able to be reestablished in HEK293T cells (Figure 17A), expression of the biosensor was not observed with proper localization after transfection, due to issues with the transfection reagent (jetPRIME®) killing off the transfected cells or causing them to be misshapen (Figure 17B). After cytoplasmic controls were reestablished in U2OS cells, transfection of the Aurora-A biosensor in U2OS cells with a new transfection reagent (ViaFect™) showed localized fluorescence at centrosomes and mitotic spindle as expected (Figure 18A). Transfections in U2OS cells were optimized and repeated with and without cell cycle synchronization. Phasor analysis of unsynchronized G1 and S phase cells and synchronized mitotic phase cells did not show distinct lifetimes that would indicate the donor and acceptor were in a FRET state, indicating that the molecule had not changed conformational state between cell cycle phases as described (Bertolin et al., 2016).
Figure 16. Aurora-A Biosensor phasor analysis in NIH 3T3 cells. (A). Lifetime images of the Aurora-A biosensor (pAurora-GFP-AURKA-mCherry) in live cells imaged at multiple cell cycle phases. Separate cells were imaged per phase. Pixels on each image represent lifetime measurements of the image with lifetime of the individual cell denoted. (B). Phasor plots for cells in panel (A). Lifetime for centrosomes contained within the red circle on plot. (C). Table with expected lifetime per cell cycle phase for active biosensor compared to observed lifetime ranges per phase. N=50 cells total within either G1, S, or mitosis expressing the Aurora-A biosensor. (D). Table with expected lifetime for kinase dead biosensor compared to observed lifetime range. N=50 cells total within either G1, S, or mitosis expressing the kinase dead version of the Aurora-A biosensor.
Figure 17. *Aurora-A Biosensor establishment in HEK293T cells.* (A). Lifetime images of the three FRET control conditions with cytoplasmic control lifetime expression as expected and observed in NIH 3T3 cells. (B). Intensity images of Aurora-A expression in HEK293T cells with cells looking unhealthy with no clear area of Aurora-A expression at centrosomes as expected.
Figure 18. **Aurora-A Biosensor establishment in U2OS cells.** (A). Lifetime images of single cells. Cell 1 in G1 and cell 2 in S phase. (B). Phasor for lifetime images. No clear difference in lifetime is present between G1 (when Aurora-A should be active=shorter lifetime) and S (when Aurora-A should be inactive=longer lifetime). Lifetime is nearly identical between the two phases indicating no change in activity of the biosensor.
After seeing a lack of FRET while using phasor analysis (Figure 19), fast-FLIM analysis was used to make a more direct comparison to the analysis methods used in Bertolin et al., 2016. In Bertolin et al., 2016 frequency domain FLIM was used for determining activity changes of the Aurora-A biosensor. Frequency domain FLIM differs from Time-Correlated Single Photon Counting (TCSPC) FLIM in that frequency domain FLIM uses a modulated light source and lifetime is determined by the phase shift and change in emission depth modulation while Time-Correlated Single Photon Counting (TCSPC) FLIM uses a laser that pulses at repeated intervals and measures the decay curve of emitted photons (van Muns ter and Gadella Jr., 2004). While the Stellaris 8 Falcon uses TCSPC FLIM and can analyze the photon decay using phasors, Bertolin et al., 2016 did not use this method for obtaining lifetime values. The nearest comparison that was able to be made to the analysis method used in Bertolin et al., 2016 was fast-FLIM analysis using fast-FLIM images produced by the Stellaris 8 Falcon, where the lifetimes within the regions of interest were averaged in ImageJ manually. While using this fast-FLIM analysis, the biosensor reported an average lifetime of 2.188 ns for G1 cells and 2.159 ns for mitotic cells, which is consistent with the published values in Bertolin et al., 2016 (Figure 19). However, this same lifetime value was also seen during S phase, when the biosensor is supposed to be in an inactive state with a longer expected lifetime. Collectively this data shows that the Aurora-A biosensor localizes to the centrosome as expected but does not show a robust change in activity of the enzyme when using Time-Correlated Single Photon Counting (TCSPC) FLIM.

Although using either method of FLIM should allow us to resolve lifetime changes caused by Aurora-A activity change, we cannot make a direct comparison between the results obtained in this study and results observed by Bertolin et al., 2016 as we are unable to use
Figure 19. Aurora-A biosensor Fast-FLIM analysis. (A) Intensity images of G1 and mitotic cells. (Yellow/white areas - highest photon count, pink areas – medium photon count, purple/blue areas - low photon count). (B) FLIM images with scale bar. N = 4 cells/stage. G1 cells average lifetime – 2.188 ns. Mitotic cells average lifetime – 2.159 ns. All images are Fast-FLIM images analyzed using imageJ pixel lifetime averaging.
frequency-domain FLIM with the current FLIM capabilities of the Stellaris 8 Falcon. Without observing a robust change in lifetime using Time-Correlated Single Photon Counting (TCSPC) FLIM, further biological questions related to the potential interaction between Pericentrin expression and Aurora-A activity could not be addressed.

**Conclusions and Future Directions**

Overall, these experiments established FRET-FLIM capabilities on the Leica Stellaris 8 Falcon, which is necessary to analyze FRET using the microscope for any future FRET-FLIM related experiments. Additionally, this work brings into question the capabilities of the Aurora-A kinase biosensor, which was unable to be established using TCSPC FLIM on the Leica Stellaris 8 Falcon. Further, while a connection between overexpression of Pericentrin and Aurora-A kinase activity was not able to be investigated, it has raised the question of if Pericentrin may act as a regulator of Aurora-A due to low rates of solid tumor formation in those with Trisomy 21 who overexpress Pericentrin. While these questions will not be further addressed by the Galati Lab, they are now open to further inquiry by others in the field.
Chapter 2: Analyzing the position and distribution of centriolar satellites

Introduction

A.1. Centriolar satellite structure and function. Centriolar satellites are membraneless granules, which move to and from the centrosome via microtubule dynein motors (Liu et al., 2010; Tollenaere et al., 2015). Centriolar satellites play roles in ciliogenesis, neurogenesis, and centrosome maintenance and maturation (Franceschini et al., 2013; Tollenaere et al., 2015). However, of the over 100 centriolar satellite components that have been discovered, most of their functions and regulation mechanisms are largely unknown (Gupta et al., 2015).

PCM1 is a 228-kDa protein and was the first centriolar satellite to be discovered (Balczon et al., 1994; Kubo et al., 1999). PCM1 is viewed as an essential centriolar satellite component and is often used to identify centriolar satellites (Hori and Toda, 2017; Tollenaere et al., 2015). Additionally, PCM1 acts as a major centriolar scaffold protein and interacts with Pericentrin (Li et al., 2001; Gupta et al., 2015a; Hein et al., 2015).

Pericentrin is a PCM component that is essential for PCM assembly (Lawo et al., 2012; Lee and Rhee, 2011; Zimmerman et al., 2004). Pericentrin exists in two distinct populations (Figure 20A). The core population resides at the centrosome, where it exists in a linear structure within the toroid that surrounds the end of the centriole (Figure 20B). The satellite population resides away from the centrosome, where it exists as dynamic granules that rapidly move to and from the centrosome via microtubules (Liu et al., 2010). During late G2/early mitosis Pericentrin near the centrosome undergoes liquid-liquid phase separation (LLPS) and forms granules around the maturing centrosome, suggesting that Pericentrin is directly involved in assembly of the centrosome via LLPS (Jiang et al., 2020).
Figure 20. Pericentrin cellular population and PCM toroid structure. (A) Pericentrin found at the centrosome and modestly throughout cell as satellites (cyan). The robust PCM1 satellite population is seen spread throughout the cell (green). Golgi (magenta) and nucleus (blue) are also shown. Imaging credit: Jonah Goodfried, Galati Lab. (B) Pericentriolar material toroid structure image taken on the Leica Stellaris 8 Falcon. Panels show Z-plane images through the toroid. Two distinct hollow rings surrounding two centrioles (hollow middle area of toroid) connected by protein linker forming one centrosome in maximum projection. PCNT (cyan) and Nucleus (Blue).
**A.2. Centriolar satellite regulation mechanism is partially unknown.** Microtubules were thought to be the contributing factor in the formation and regulation of centrosomes and their satellites. This is due to centrosomes being the major microtubule organizing center in cells (Efimov et al., 2007). However, when microtubules are disrupted, centrosomes remain intact, and centriolar satellites cluster in groups throughout the cell (Meraldi and Nigg, 2001). The lack of complete dispersal of satellites throughout the cell after microtubule disruption is indicative of a secondary mechanism that regulates centrosomes and their satellites alongside microtubules.

**A.3. Proposed model for centriolar satellite regulation.** During mitosis, centriolar satellites undergo dissolution and dissipate throughout the forming cells. However, the satellites reassemble to their pre-mitotic state once mitosis is complete (Kubo and Tsukita, 2003). Interestingly, during mitosis the Golgi also disperses rather than remaining intact throughout mitosis (Colanzi and Sütterlin, 2013; Sütterlin et al., 2002). Due to the similar cell cycle-dependent distribution patterns of centriolar satellites and the Golgi and the microtubule-independent localization of centriolar satellites, we propose that the Golgi regulates the distribution of centriolar satellites (Figure 21). To test this prediction, GFP-PCNT RPE cells were exposed to the drug Brefeldin-A (BFA), which disperses the Golgi into small vesicles. The distribution of centriolar satellites was investigated by using live cell confocal microscopy to compare the position of centriolar satellites before and after Golgi dispersal.
Figure 21: Proposed model of satellite dispersal after Golgi disruption. Model shows Golgi satellite population dispersing alongside the Golgi after addition of BFA while centrosome satellite population remains largely unchanged. When centrosomes are away from the Golgi a secondary population of satellites is seen colocalized with the Golgi. This population is proposed to be regulated, in part, by the Golgi. When the Golgi is disrupted, we anticipate the satellites dispersing alongside the Golgi. Created in BioRender.
**Materials and Methods:**

**B.1. Cell Culture.** hTERT-Retinal Pigmented Epithelial (RPE) cells stably expressing mNeon-Pericentrin (mNeon-PCNT) (McCurdy et al., 2022) were cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) and 10 mL/L Penicillin/Streptomycin/Fungizone Solution 100X, HyClone™ (Cytiva). The cells were kept at 37°C in a 5% CO₂ environment.

**B.2. Pharmacology.** Cells were exposed to +/- Aphidicolin (Adipogen), a drug that synchronizes cells at the G1/S boundary, once coverslips reached approximately 80% confluency. The cells were incubated in 5 ng/μL of +/- Aphidicolin (Adipogen) for 18-20 hours before imaging. A two-hour washout of the drug was performed to allow the cells to reach S phase for imaging (Bertolin et al., 2016).

Brefeldin A (BFA) is fungal macrocyclic lactone that breaks down the Golgi and redistributes it into the endoplasmic reticulum (Chardin and McCormick, 1999; Nebenführ et al., 2002). BFA (BioVision) was reconstituted in DMSO and used at a final concentration of 0.55 μM to disperse the Golgi during the live cell imaging experiments.

**B.3. Confocal Microscopy** hTERT-Retinal Pigmented Epithelial (RPE) cells stably expressing mNeon-Pericentrin (mNeon-PCNT) were split onto coverslips approximately 72 hours before imaging. The cells were then incubated in 2.5 μM SiR Tubulin (Cytoskeleton) for 18-20 hours which stains endogenous microtubules for imaging (“SiR-tubulin kit - Live cell microtubule probe,”). Two hours before imaging the coverslips were washed with FluoroBrite™ DMEM supplemented with 10% FBS, then incubated for 10 minutes in media containing BODIPY™ TR Ceramide complexed to BSA (Invitrogen™) at 0.5 μM for both ceramide and BSA. After an
additional washout into FluoroBrite™ DMEM the cells were imaged in the stain free media. After the full 2-hour washout period from Aphidicolin +/-, cells were imaged once every 15 minutes for 30 minutes before the addition of 0.55 uM Brefeldin A reconstituted in DMSO (BioVision 150-5) or the same volume of DMSO and again every 15 minutes for one hour after the addition of the drug or control.

Confocal microscopy live cell images were taken using the Leica Stellaris 8 Falcon FLIM microscope with a 63X 1.4 NA objective. Cells were kept in at 37°C in a 5% CO₂ environment using a Tokai Hit stage incubator. Images were taken using excitation wavelengths of 489, 595, and 652. Scan speed was set to 8000 Hz in a format of 1024 x 1024 with 2-fold frame averaging and 4-fold line averaging. Images were collected at 0.4-micron Z steps. For deconvolution Leica LASX Lightning Software V4.2 was used. ImageJ software was used to produce maximum projections of the stacked images.
Results and discussion:

C.1. Characterization of cell cycle dependent centrosome dynamics. As cells are dynamic and exhibit different features based upon cell cycle stage, we set out to characterize cell cycle dependent changes in centrosomes (PCNT marker), centriolar satellites (PCNT and PCM1 markers), and the Golgi in RPE cells. Both PCM1 and Pericentrin markers were labeled in fixed cells as PCM 1 has a robust satellite population, while Pericentrin has duality with both a core population and modest satellite population (Figure 22). During G1, cells have a single centrosome and fully intact Golgi. By G2, the centrosomes have duplicated, but the Golgi remains intact. During interphase, we observed that in cells where centrosomes are separated spatially from the Golgi, there are two distinct populations of satellites within the cell. The first population surrounds the centrosome as expected. The second population is distributed at and near the Golgi (Figure 22).

During mitosis, the Golgi begins to disassemble and spread throughout the cell. By anaphase, the Golgi has dissipated throughout the cell, leaving no identifiable Golgi structure. Interestingly, this pattern is also seen with the distribution of satellites during mitosis, where they are also dispersed almost evenly throughout the dividing cells (Figure 22). As an interaction between the Golgi and centriolar satellites has not yet been established, observation of a similar distribution pattern of both cellular components warranted further inquiry of a potential association between them.

C.2. Cell categorization based upon centrosome and Golgi phenotype. To determine whether Golgi distribution impacts the spatial organization of centriolar satellites, GFP-PCNT RPE cells were exposed to the Golgi disruptor drug Brefeldin-A (BFA). BFA breaks apart the Golgi into
**Figure 22. Cell cycle satellite and Golgi dispersal.** Imaging credit: Jonah Goodfried. Satellite and Golgi distribution during interphase and mitosis in fixed PCNT-RPE cells. Blue- Nuclei, Magenta-Golgi, Teal- Pericentrin, Green- Pericentriolar Material 1 (PCM1). Magenta lines indicate areas of congregated Golgi, teal boxes indicate centrosomes, and green box and/or lines indicate areas of PCM1 localization. Zoom in scale bar = 1 um. White boxes on composite image show areas of zoom in location where one area overlays a centrosome and the other overlays a portion of the Golgi.
small vesicles from its larger intact structure. In RPE cells, 0.55 uM BFA fully disrupts the Golgi after a 30-minute incubation at 37°C in a 5% CO₂ environment. BFA does not impact centrosomes which remain intact (Figure 23). As the Golgi begins to break apart during mitosis, these experiments needed to occur during interphase while the Golgi was intact to determine potential effects of Golgi dispersal. However, due to the modest population of Pericentrin satellites, the cells were synchronized in S phase to increase levels of Pericentrin present while the cells remained in interphase.

During S phase these cells can be categorized into four phenotypes based upon the number of centrosomes present within a cell and the centrosomes association status with the Golgi (Figure 24). Cells can be classified first as either centrosome associated, where there is one visible centrosome present in the cell, or centrosome separated, where there are two distinct centrosomes visible in the cell. Those centrosomes are then categorized individually relative to their association with the Golgi. A centrosome is considered associated with the Golgi if it is overlayed with it on the image or separated from the Golgi if the centrosome is not overlayed on the Golgi. This leaves four categories as follows: Centrosome associated/Golgi associated, Centrosome associated/Golgi separated, Centrosome separated/Golgi associated, and Centrosome separated/Golgi separated (Figure 24). Cells were categorized based upon their initial centrosome number and Golgi position before the addition of BFA.

C.3. Golgi Disruption affects centriolar satellite distribution: After a 30-minute DMSO incubation, the control group of centriolar satellites exhibited minor changes when compared to before introduction of DMSO. However, the changes were expected due to cells being dynamic during live cell imaging. The overall changes were extremely minimal where the satellites seemingly remained in their original placement throughout the cell and their intensity also
Figure 23. BFA disrupts the Golgi in GFP-PCNT RPE cells. Timelapse of the same cells with Pericentrin (green) and Golgi (magenta) indicated. Golgi is fully dispersed at 30 minutes after the addition of BFA to cells. This effect is not observed in the control cells exposed to DMSO.
Figure 24. Cells in S phase are categorized based upon the number of centrosomes present and their relative position to the Golgi. Upper left: Centrosome associated/Golgi associated. Upper right: Centrosome separated/Golgi associated. Lower left: Centrosome associated/Golgi separated. Lower right: Centrosome separated/Golgi separated.
remained the same (Figure 25). However, in the cells exposed to a 30-minute BFA incubation, the centriolar satellites exhibited a larger change to their satellite population distribution based upon the centrosome, Golgi, and satellites positioning within the cell (Figure 25). In a randomized set of 12 images taken from across multiple imaging days, there is a clear difference visually that Golgi disruption affects the distribution of these satellites differentially based upon different Golgi, centrosome, and satellite positioning. In cells with associated centrosomes that are not associated with the Golgi, when the Golgi is fully dispersed, satellites that were once associated with the Golgi disperse (Figure 26). As seen in the image taken 15 minutes before the addition of BFA, the satellites congregate in the white outlined area within the cell. Thirty minutes after the addition of BFA, the satellites that were once in that area dissipate as marked by the red outlined area on the image (Figure 26). In cells with associated centrosomes that are associated with the Golgi, when the Golgi is dispersed there is no visual change in the satellite population, and it is assumed that the satellites remain associated with both the centrosome and Golgi (Figure 27). In the image taken before the addition of BFA, the white outlined area remains very similar in size and intensity surrounding the centrosome before and after the addition of BFA indicating no change in satellite distribution or intensity with disruption of the Golgi (Figure 27). In cells with separated centrosomes where both centrosomes are associated with the Golgi, after the Golgi is dispersed, the satellites remain associated with both the centrosome and the Golgi (Figure 28). As seen in the image taken before the addition of BFA compared to 30 minutes after BFA, the white outlined areas remain the same in size and intensity, again indicating little to no change in satellite distribution with Golgi disruption (Figure 28). In cells with separated centrosomes where at least one centrosome is separated
Figure 25. Golgi disruption affects centriolar satellite position and distribution in RPE cells. Raw images from BFA timelapse experiment showing Pericentrin localization and intensity. Top row of BFA or DMSO cells used as a control for “normal” distribution of satellites and centrosomes for individual cells with populations outlined in white. Bottom row of either BFA or DMSO cells 30 minutes after the addition of BFA or DMSO with white outlines indicating no noticeable change to the distribution of satellites while red indicates a change in distribution of intensity of the satellites at that given position within the cell.
**Figure 26. Distribution of satellites in a cell where centrosomes are associated and are separated from the Golgi.** Image of cell 15 minutes before the addition of BFA is used as the baseline distribution for centrosome and satellites with boundary of major satellite population outlined in white. Image 30 minutes after the addition of BFA has same area where satellites were present in initial image outlined in red as there is a visual difference in intensity and distribution of satellites in that area.
Figure 27. Distribution of satellites in a cell where centrosomes are associated with one another, and both are associated with the Golgi. Image of cell 15 minutes before the addition of BFA is used as the baseline distribution for centrosome and satellites with boundary of major satellite population outlined in white. Image 30 minutes after the addition of BFA has same area where satellites were present in initial image outlined in white as there is no visual difference in intensity and distribution of satellites in that area indicating a lack of change in satellite distribution after the addition of BFA.
Figure 28. Distribution of satellites in a cell where centrosomes are separated from one another, and both are associated with the Golgi. Image of cell 15 minutes before the addition of BFA is used as the baseline distribution for centrosome and satellites with boundary of major satellite population outlined in white. Image 30 minutes after the addition of BFA has same area where satellites were present in initial image outlined in white as there is no visual difference in intensity and distribution of satellites in that area indicating a lack of change in satellite distribution after the addition of BFA.
Figure 29. Distribution of satellites in a cell where centrosomes are separated from one another and at least one of the centrosomes is separated from the Golgi. Image of cell 15 minutes before the addition of BFA is used as the baseline distribution for centrosome and satellites with boundary of major satellite population outlined in white. Image 30 minutes after the addition of BFA has a red outlined area showing the change in the satellite distribution after the addition of BFA where most of the satellites are dispersed in other areas of the cell.
from the Golgi, the Golgi satellites seemingly disperse alongside the Golgi when disrupted (Figure 29). The satellites outlined in white before the addition of BFA dissipate after as indicated by the red outline on the image after Golgi dispersal (Figure 29). While some change would be expected between images before and after the addition of BFA due to normal fluctuations within live cells, these changes were not as drastic in the group of cells exposed to DMSO. This indicates that the changes seen when the Golgi is dispersed are not normal fluctuations within the cell, and rather an effect caused by Golgi dispersal. While further details of how this may affect cells in the long term have not yet been addressed, this does point to a relationship between the regulation of the Golgi and satellites.

Differences in the distribution of satellites between the cell categories suggest that the effect of Golgi disruption on centriolar satellites is not an all or nothing phenotype. Rather, certain categories of cells respond differently to the effects of Golgi disruption. In a small number of interphase cells the Golgi and centrosome are not colocalized. These cells exhibit centrosome separation from the Golgi and have two populations of centriolar satellites, where one population is clustered at or near the Golgi and the other at or near the centrosome. In our model (Figure 30) we propose that in this specific subset of cells in which the Golgi is separated from the centrosome, the Golgi acts as a secondary regulator of satellite localization of only that subset of satellites. This is due to the observation that when the Golgi is disrupted, only the Golgi satellites disperse throughout the cell, while the centrosome associated satellites remain in their prior location. However, in cells where the Golgi and centrosome are colocalized, there is a single satellite population encompassing both the centrosome and Golgi. This population remains unchanged by the effects of Golgi disruption. This suggests that there are at least two layers to the control and regulation of centriolar satellites. First, we suggest that centrosome-based control
of the satellites acts as the primary control course over satellite positioning and overshadows the Golgi control mechanism when the two organelles are colocalized. Secondly, only when the Golgi is separated from the centrosome does a secondary control mechanism regulated by the Golgi come into play due to the lack of centrosome control within that area of the cell. This potential regulatory mechanism provided of the Golgi may tie into the known relationship between cilia and the Golgi. Golgi-localized proteins have been implicated in ciliogenesis and IFT20 (Intraflagellar Transport 20) localizes to the Golgi, basal body, and cilium (Asante et al., 2013; Follit et al., 2006). This further supports the idea that the Golgi assists in the regulation of a subpopulation of centriolar satellites.


While a phenotypic result has been visually identified, quantification of the phenotype is complex, and a working quantification method has yet to be developed. Prior methods of analyzing satellite dispersal were simply visually scored as either dispersed or not dispersed, heavily biasing the analysis. Creating a quantification method for satellite dispersal would enable less biased analysis within the field.

Attempts at quantification began using concentric boxes centered around a single centrosome, where intensity was measured and averaged per pixel for concentric areas on the image. However, this methodology was not effective due to the signal to noise ratio of the images, where the background noise overshadows the dim signal from the satellites. This left images with visual differences in satellite distribution, but without a quantitative difference between the phenotypes. Attempts at quantification of the satellite phenotypes will continue as we push toward publication of results.
While live cell imaging allows for the comparison of the same cells over time, quantifying small, dim structures with live cell imaging is challenging. Live cell imaging forced us to reduce laser power and increase the speed of the laser scanning to keep the cells healthy during imaging. This reduced the maximum intensity of Pericentrin within the images to between 1-15 photons, which makes it difficult to quantify. This is very minimal intensity compared to what can be produced in fixed cell imaging, where we are able to increase laser intensity and decrease the scan speed without fear of killing the cells. In the future, these experiments will likely be repeated using fixed cells to confirm the phenotypic results seen within this dataset. This will also provide a secondary dataset with more power for quantification of these phenotypes.

**Conclusions and Future Directions**

While changes in centriolar satellites after Golgi disruption do not occur in every cell phenotype, within the subset that the Golgi and centrosome are separated, the Golgi plays a role in the regulation of a subset of Golgi associated satellites. This indicates that the Golgi acts as a secondary regulator of centriolar satellites when they are separated from the centrosome. This work is the first to investigate a potential regulatory mechanism for centriolar satellites involving the Golgi and has shown that cells exhibit phenotypic differences after Golgi disruption rather than an all or nothing phenotype. While the signal to noise ratio of the live cell imaging performed in these experiments limited quantification capabilities using this dataset, further analysis and quantification of the phenotypes are currently being investigated and developed by other members of the Galati Lab in ongoing experiments.
Figure 30. Model of effect of Golgi distribution of centriolar satellite based upon centrosome and Golgi positioning. Satellites in cells with centrosomes associated with the Golgi experience no change in centriolar satellite distribution after Golgi disruption. In cells with centrosomes separated from the Golgi, Golgi disruption causes the Golgi population of centriolar satellites to disperse. Golgi – magenta, centrosomes- teal, centriolar satellites – teal rounds, Golgi vesicles – magenta rounds.
Chapter 3: Characterization of \textit{C. elegans} autofluorescence using spectral scanning and Fluorescence Lifetime Imaging microscopy (FLIM)

\textbf{Introduction}

\textit{A.1. Caenorhabditis elegans} (\textit{C. elegans}) is a model organism with autofluorescence. \textit{C. elegans} is a transparent nematode that is widely used as a model organism for studying cellular processes related to development and disease. \textit{C. elegans} has been used in studies related to Parkinson’s, Alzheimer’s, pathogen infection and immunity (Balla and Troemel, 2013; Ewald and Li, 2010; Harrington et al., 2010). Many of these studies require creating transgenic lines of reporters for various proteins and components of interest in these diseases, often tagged with common fluorescent proteins like GFP or mCherry (Kapulkin et al., 2005; Perni et al., 2017; Richardson et al., 2010). However, as \textit{C. elegans} has background autofluorescence that is spectrally similar to GFP and mCherry, using fluorescent proteins and dyes with similar fluorescence spectra is problematic for distinguishing true fluorescence from background (Pincus et al., 2016). This is especially apparent when the fluorescent reporters are expressed near the cuticle and gut of the organism where the background autofluorescence of the nematode is most prominent (Teuscher and Ewald, 2018).

\textit{A2. Proposing Phasor-FLIM as a novel method for characterizing autofluorescence in \textit{C. elegans}.} Quantitatively, background subtractions and thresholding are not ideal for the removal of background caused by the nematodes autofluorescence, especially in transgenic lines that have very low fluorescence intensity. However, fluorescence lifetime can be used to differentiate \textit{C. elegans} autofluorescence from fluorescence of fluorescent molecules with similar spectra. This method has not yet been used to characterize autofluorescence in \textit{C. elegans}. Phasors are a robust
way to characterize and quantify lifetime of these auto fluorescent areas of the nematode, without the use of curve fitting. Combined Phasor-FLIM has been used to characterize fluorescence in several biological contexts. These studies include characterizing changes in protein folding and activity using FRET-FLIM (Bertolin et al., 2016; Ng et al., 1999), characterizing changes in lifetime of NADH enzymatic binding activity (Gómez et al., 2018), and clinically FLIM is used in detecting inflammation and tumor boundary delineation (Ouyang et al., 2021).

A.3. Can C. elegans autofluorescence be characterized using Phasor-FLIM? The goal of this experiment was to determine whether Phasor-FLIM could be used to distinguish background from the C. elegans gut and cuticle from fluorescence of spectrally similar fluorescent molecules and dyes. The lifetime of the gut and cuticle of WT nematodes was investigated, and it was determined that Phasor-FLIM provides a reliable and robust way to differentiate the detection of low intensity GFP from auto fluorescent regions of the nematode.
Materials and Methods:

B.1. C. elegans preparation (Performed by Heino Hulsey-Vincent in the Dahlberg Lab).

Worms are grown on neutral growth media (NGM) plates, which have been spotted with OP-50 E. coli and stored in a 21.5C incubator. Four days before imaging, 5 gravid C. elegans are put into 15uL of bleach mix (500uL bleach, 400uL 5M NaOH, 100uL H2O). Slides are prepared by pipetting 70 uL of molten agarose onto a glass slide and placing an additional glass slide on top to form an agarose pad. Then 8 uL of 30mg/mL BDM is pipetted onto a coverslip and covered with a petri dish lid. Then 8-12 four-day old worms are then pipetted into the BDM droplet and incubated for 15 minutes while covered. Agarose pad/glass slide are then placed on coverslip for imaging.

B.2. Spectral scanning. Single plane emission scans were taken using the 63X 1.4 NA oil immersion objective attached to an automated Leica Stellaris 8 laser scanning confocal microscope. A laser line for the 405 DAPI diode at 405 nm, preset eGFP spectra at 473 nm, preset Alexa 568 spectra at 561 nm, and laser line at 647 nm were used in the emission scan with a constant laser intensity of 50. Emission spectra were taken across a single plane through the midpoint of the worm. For each excitation, emission spectra were collected every 30 nm. Ranges of collection are as follows: 420-780 nm for the 405 nm laser line, 480-780 nm for the 473 nm laser line, 570-780 nm for the 561 nm laser line, and 660-780 for the 647 nm laser line.

B.3. FLIM Microscopy. C. elegans were imaged using the 63X 1.4 numerical aperture oil immersion objective attached to an automated Leica Stellaris 8 laser scanning confocal microscope. Both WT and GFP expressing worms were excited with the 488 nm laser line of an 80 MHz pulsed white light laser and fluorescent photons were collected using a time-correlated
single photon counting device. Decay traces of photon arrival times were analyzed using the Phasor approach through the Falcon - FLIM module of the Leica LasX software package.
Results and Discussion:

C.1. Spectral scans of WT C. elegans autofluorescence. Spectral scans characterizing the autofluorescence of WT C. elegans showed varying amounts and locations of autofluorescence within the nematode. At an excitation of 405 nm, the worm exhibits high amounts of autofluorescence within the gut granules of from 420 to 690 nm and a lesser amount within the cuticle from 480 to 690 nm (Figure 31). At an excitation of 473 nm the cuticle and gut granules both emit autofluorescence from approximately 480 to 690 nm. The pixel intensity in the areas of autofluorescence also decreases as emission wavelength increases (Figure 31). At an excitation of 561 nm the autofluorescence is less prominent than at 405 and 473, with minor gut granule autofluorescence emitted from 570 to 750 nm (Figure 31). Lastly, at an excitation of 647 nm autofluorescence is extremely minimal with little to no signal present from 660 to 780 nm emission making the areas of the worm exhibiting the autofluorescence difficult to distinguish (Figure 31). Due to the low intensity of the autofluorescence at 561 and 647 nm excitations, further analysis of C. elegans at these spectra were not pursued.

C.2. Spectral scans reveal unique populations of gut granule autofluorescence. Gut granule autofluorescence is generally present at all spectral scan excitations, with autofluorescence intensity decreasing as emission increases. Additionally, there are three prominent populations of gut granules that can be characterized from the spectral scans. The first population of autofluorescence present in the gut is only observed at a 405 nm excitation (Figure 32). These are thought to be yolk granules which emit autofluorescence from 420 to 630 nm. The second population of autofluorescence can be excited at both 405 nm and 473 nm (Figure 32). These gut granules emit autofluorescence with an excitation
Figure 31. Spectral scan of WT *C. elegans* background autofluorescence at 405, 473, 561, 647 nm excitation. All images obtained from single worm. Excitation of WT *C. elegans* with excitation spectra matching Alexa 405, GFP (473), 561, and 647 with emission captured at listed wavelengths in bottom right corner of the image. Emission scale bar shows color based on emission nm of autofluorescence. White areas indicate emission within blue, green, and red spectra. Image scale bar 50 um.
Figure 32. Spectral scan of WT *C. elegans* gut granule autofluorescence at 405, 473, 561, 647 nm excitation. DIC image contains red box which matches area of gut granule spectral scan. All images obtained from single worm. Excitation of WT *C. elegans* with excitation spectra matching Alexa 405, GFP (473), 561, and 647 with emission captured at listed wavelengths at the bottom of each image. Emission scale bar shows color based on emission nm of autofluorescence. White areas indicate emission within blue, green, and red spectra. Image scale bar 5 um.
of 405 nm from 420 to 750 nm and with an excitation of 473 from 480 to 660 nm. Within this spectra, granules of two sizes are present. One population consists of larger granules and a separate population consists of smaller granules. These gut granule populations are found throughout multiple regions of the gut. Together these spectral scans indicate that different gut granule species have different spectral characteristics that can be distinguished solely using spectral scanning. To further identify if these populations of gut granules at the same excitation can be distinguished from one another, Phasor-FLIM was used at 473 nm. Phasor-FLIM analysis at 405 nm was not pursued due to lack of a pulsed 405 laser limiting our FLIM capabilities.

**C.3. Phasor-FLIM characterization of gut granule autofluorescence at 473 nm shows three lifetime populations.** Initial Phasor-FLIM analysis of the gut granules at 473 nm excitation showed three populations of lifetime within the gut granules (Figure 33). Each granule population was centered on a separate location on the phasor plot. These populations exist throughout multiple areas of the gut. The difference in lifetime populations may also reveal biochemical distinctions within the gut granules. This difference in location on the Phasor allows for further characterization than typical spectral scanning. Additionally, within a subset of these gut granules some have subregions with distinct lifetime populations (Figure 34). These lifetime differences may also indicate that within gut granules there are biochemically distinct regions. These biochemically distinct granules are also present within multiple regions of the gut. This additional ability to characterize autofluorescence in a lifetime-based fashion highlights the inability of spectral scanning to fully identify and characterize autofluorescence. However, Phasor-FLIM provides an additional robust way to further identify and characterize these autofluorescence populations based upon photon decay rate.
Figure 33. WT *C. elegans* autofluorescence characterized using FLIM at 473 nm excitation. **Fast-FLIM:** (Top) Representative image of *C. elegans* indicating the approximate locations of images taken in the gut. (Middle) Fast-FLIM images show gut granules with a color scale for the average photon decay time per pixel within the image. (Bottom) The phasor includes two individual image phasor plots combined. The area to the right of the phasor plot corresponds with shorter photon arrival time and areas on the left corresponds to longer photon arrival time. The color of circle on the phasor plot corresponds to the colored overlay on phasor masked images.
**Figure 34. A subset of gut granules have subregions with distinct lifetime populations.** Images were taken in two gut areas from a single animal, as in Figure 3. (Middle) Fast-FLIM images show gut granules with a color scale of the average photon decay time per pixel within the image. (Bottom, right) The phasor includes two individual image phasor plots combined. The area to the right of the phasor plot corresponds with shorter photon arrival time and areas on the left correspond to longer photon arrival time. The color of circle on phasor plot corresponds to the colored overlay on phasor masked images.
C.4. Phasor-FLIM can distinguish GFP fluorescence from C. elegans autofluorescence. In addition to autofluorescence being characterized using Phasor-FLIM, GFP fluorescence can be distinguished from the autofluorescence using Phasor-FLIM analysis. Using the same 473 nm excitation in a strain of C. elegans with dim neuronal GFP expression, the GFP expressing neuron has a lifetime of 2.676 ns while the auto fluorescent cuticle exhibits a lifetime of 1.003 ns (Figure 35). The lifetime of the GFP neuron is near the expected lifetime of GFP of around 2.4 ns confirming that the lifetime population is accurately detecting GFP. This can be replicated in neurons expressing both high and low levels of GFP (Figure 36). Using Phasor-FLIM masking overlays onto intensity images, lifetime-based regions of interest can be “extracted” from the images, leaving behind only the portions of the image that are within the intended phasor space.

Conclusions and Future Directions:

Being able to distinguish between the dimly expressing GFP tagged neuron and autofluorescence is important as C. elegans neurons are often used in studies involving neural development and function. Dim expression of tagged proteins within the neuron has proven to be challenging to distinguish from the autofluorescence (Spencer et al., 2014). By using Phasor-FLIM it can be easily accomplished, and neurons or other protein tagged areas within the nematode can be easily separated from the autofluorescence. In addition to the lifetime separation observed with Phasor-FLIM, in areas outside of the neuron or cuticle, the photon count is very low and can be easily removed using thresholding to remove additional background noise. Using this methodology, further studies using the model organism C. elegans will be able to distinguish between background autofluorescence of the nematode and fluorescence of commonly used fluorescent
proteins and dyes. In the future we plan to continue this work by determining if Phasor-FLIM can be applied with different fluorescent proteins and dyes in other areas of the worm and further investigate the potential biochemical distinctions within the different granules and lifetime subregions.
Figure 35. GFP tagged neuron fluorescence and auto fluorescent background characterization in *C. elegans*. **Fast-FLIM**: Fast-FLIM image depicting mouth area of *C. elegans* with neuron in yellow/red and cuticle and background in green/blue. **Fast-FLIM with Phasor Overlay**: Phasor overlay for two populations of photons. Left image with overlay for cuticle and general background fluorescence with a lifetime of 1.003 ns. Right image with overlay for neuron with a lifetime of 2.676 ns. **Phasor**: phasors for corresponding images as depicted above.
Figure 36. GFP can be distinguished and isolated from autofluorescence that is excitable at 473 nm. Max projection: maximum projection of mouth area of *C. elegans* with varying intensity of GFP expressing neuron. Fast-FLIM: Fast-FLIM image depicting mouth area of *C. elegans* with neuron in and cuticle and background in green/blue. Lifetime scale bar below. Phasor: Phasor corresponding to Fast-FLIM image to the left of phasor. Phasor Masks: Phasor masks correspond to colored circle area on Phasor. Yellow – neuron, cyan- cuticle. “Extracted”: Areas of overlay from Phasor masked images correspond to areas “extracted”.

High GFP

Low GFP

No GFP

Max Projection Fast FLIM Phasor Plot Phasor Masks Neuron “Extracted” Cuticle “Extracted”
Chapter 4: Applications of this study for future research and medical advancements

Fluorescence Lifetime Imaging Microscopy (FLIM) allows us to gain a deeper understanding of the physical properties of biological samples. Not only is FLIM heavily used in biological sciences, but the technique spans multiple fields and is increasingly being utilized for a wider range of applications. Of the many biomedical applications of FLIM, it is notably used for cancer therapeutic development and cancer treatment. Optical redox ratios were once discussed as a potential method for the diagnosis of oral and cervical cancers, but the method is not sensitive enough due to high background compared to the low quantum yield of NAD(P)H and FAD (Gnanatheepam et al., 2020; Jing et al., 2018; “Label-free imaging and spectroscopy for early detection of cervical cancer - Jing - 2018 - Journal of Biophotonics - Wiley Online Library,” ). However, FLIM can be used to monitor metabolism using the intrinsic fluorescent and nonfluorescent properties of NADH/NAD(P)H as well as FAD/FADH$_2$. This is due to non-protein bound NAD(P)H having a shorter lifetime than protein bound NAD(P)H. Protein bound FAD also has a shorter lifetime than non-protein bound FAD (Blacker et al., 2013). Due to most cancer cells preference for glycolysis rather than oxidative phosphorylation, the observed lifetime of these tissues is shorter than that of the surrounding healthy tissues. This difference in lifetime allows surgeons to distinguish between tissue types during surgery, especially tumor excisions, using FLIM. This tool allows surgeons to have real-time feedback during the surgeries rather than relying on subjective evaluation of tissues (Alfonso-Garcia et al., 2020).

In addition to FLIM’s uses in the cancer field, Aurora-A is being studied as a potential target for cancer therapeutics. In some cancers, overexpression of Aurora-A correlates with diminished survival, making it a promising prognostic biomarker (Goos et al., 2013; Landen et al., 2007; Lassmann et al., 2007; Wang et al., 2011). Many small molecule inhibitors of Aurora-
A have been developed, but few of which are selective for Aurora-A (Malumbres and Pérez de Castro, 2014). Aurora-A is overexpressed in many tumor types including ovarian tumors (Landen et al., 2007; Lassmann et al., 2007), gastrointestinal tumors (Goos et al., 2013), squamous cell carcinomas (Reiter et al., 2006), among many more. Currently, the only drug that has made it to phase III clinical trials as an Aurora-A inhibitor is MLN8237 (alisertib), which was used within my thesis experiments to inhibit Aurora-A activity (Malumbres and Pérez de Castro, 2014). The phase III trial of MLN8237 for patients with relapsed or refractory peripheral T-cell lymphoma was discontinued as the drug did not provide superior progression-free survival. However, the drug is continuing to be investigated for use in small cell lung cancer (“Takeda Announces Termination of Alisertib Phase 3 Trial in Relapsed or Refractory Peripheral T-cell Lymphoma,”). Although Aurora-A is an important therapeutic target, no Aurora-A related therapeutics have been FDA approved for treatment. Although the proposed Aurora-A related experiments within this thesis were unable to be completed, the potential for these experiments to be repeated using a more sensitive Aurora-A biosensor would benefit our understanding of Aurora-A and other Aurora-A pathway components. As proposed in this thesis, if Pericentrin or other Aurora-A pathway components can regulate Aurora-A expression, new potential targets for cancer therapeutics or ways to inhibit Aurora-A overexpression may be revealed. This may lead to advancements for potential cancer treatments and new targets for cancer drugs.

Like Pericentrin, many centriolar satellite proteins play major functions during mammalian development and homeostasis. Recently CCDC11 (coiled-coil domain containing 11) was discovered to play a role in body patterning in zebrafish. When CCDC11 is depleted, zebrafish exhibit asymmetric L-R body patterning (Silva et al., 2016). Additionally, CCDC13 (coiled-coil domain-containing protein 13) interacts with PCM1, Pericentrin, and Cep290.
CCDC13 was found to prevent accumulation of DNA damage during mitosis. This discovery broadens the known functions for centriolar satellites to include roles in DNA damage prevention and maintaining genome stability (Staples et al., 2014).

In addition to the importance of centriolar satellites for regulation of cell division and DNA damage accumulation prevention, relationships between organelles are important for cellular regulation. Relationships between organelles have previously been studied in many contexts. This includes the connection between the endoplasmic reticulum and the mitochondrial membrane and the connection between the mitochondria and plasma membrane (Islinger et al., 2015). However, a tie between centriolar satellites and the Golgi has never been investigated. As centrosomes and their satellites are extremely important for maintaining proper cell division, investigating if there is a tie between centriolar satellites and the Golgi is important for understanding how the two organelles are regulated and what factors may influence changes in the organelles.

Overall, this study has both addressed novel biological concepts and broadened uses of Phasor-FLIM. This study has many human health implications as Aurora-A, Pericentrin, and many other centrosome and satellite proteins are associated with human disease. By using the techniques present in this thesis to study how these centrosome proteins and organelles are regulated, we can better understand the mechanisms by which they cause disease. Additionally, the novel Phasor-FLIM technique related to autofluorescence characterization can now be used by other C. elegans researchers to distinguish fluorescence of proteins and dyes from autofluorescence of the nematode.
Citations


